A temperature sensitive live-attenuated canine influenza virus H3N8 vaccine

Aitor Nogales[a], Laura Rodriguez[a], Caroline Chauché[b], Kai Huang[c], Emma C Reilly[d], David J. Topham[d], Pablo R. Murcia[b], Colin R. Parrish[c], and Luis Martínez-Sobrido[a]*

[a] Department of Microbiology and Immunology, University of Rochester, Rochester, New York, US.

[b] MRC-University of Glasgow Centre for Virus Research, Glasgow, United Kingdom.

[c] Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, US.

[d] David H. Smith Center for Vaccine Biology and Immunology, University of Rochester, Rochester, New York, US. Department of Microbiology and Immunology, University of Rochester, Rochester, New York, US.

[*] To whom correspondence should be addressed:
Luis Martínez-Sobrido
Department of Microbiology and Immunology
University of Rochester School of Medicine and Dentistry
601 Elmwood Avenue, Rochester, NY 14642
Tel.: (585) 276-4733
e-mail: luis_martinez@urmc.rochester.edu

Running title: A live-attenuated canine influenza virus H3N8 vaccine

ABSTRACT

Canine influenza is a respiratory disease of dogs caused by canine influenza virus (CIV). CIV subtypes responsible for influenza in dogs include H3N8, which originated from the transfer of H3N8 equine influenza virus to dogs; and the H3N2 CIV, which is an avian-origin virus that adapted to infect dogs. Influenza infections are most effectively prevented through vaccination to reduce transmission and future infection. Currently, only inactivated influenza vaccines (IIVs) are available for the prevention of CIV in dogs. However, the efficacy of IIVs is suboptimal and novel approaches are necessary for the prevention of disease caused by this canine respiratory pathogen. Using reverse genetics techniques, we have developed a live-attenuated CIV vaccine (LACIV) for the prevention of H3N8 CIV. The H3N8 LACIV replicates efficiently in canine cells at 33^{o}C but is impaired at 37-39^{o}C temperatures, and was attenuated when compared to wild-type H3N8 CIV, *in vivo* and *ex vivo*. The LACIV was able to induce protection against H3N8 CIV challenge with a single intranasal inoculation in mice. Immunogenicity and protection efficacy were better than that observed with a commercial CIV H3N8 IIV, but provided limited cross-reactive immunity and heterologous protection against H3N2 CIV. These results demonstrate the feasibility of implementing a LAIV approach for the prevention and control of H3N8 CIV in dogs and suggest the need for a new LAIV for the control of H3N2 CIV.

IMPORTANCE

Two influenza A virus subtypes has been reported in dogs in the last 16 years - the canine influenza virus (CIV) H3N8 and H3N2 of equine and avian origin, respectively. To date, only inactivated influenza vaccines (IIVs) are available to prevent CIV infections. Here we report the generation of a recombinant, temperature sensitive H3N8 CIV as a live-attenuated influenza vaccine (LAIV), which was attenuated in mice and dog tracheal, explants when compared to CIV H3N8 wild-type. A single dose of H3N8 LACIV showed immunogenicity and protection against a homologous challenge that was better than that conferred with an H3N8 IIV, demonstrating the feasibility of implementing a LAIV approach for the improved control of H3N8 CIV infections in dogs.

INTRODUCTION

Influenza A viruses (IAVs) are enveloped viruses that belong to the *Orthomyxoviridae* family, and contain a genome that comprises eight single-stranded negative-sense RNA segments that encode for 10–14 proteins (1). The hemagglutinin (HA) and the neuraminidase (NA) glycoproteins are the major antigenic determinants of IAV and are essential for receptor binding and fusion, and virion release, respectively (2). IAV HA and NA glycoproteins within infected organisms and populations are driven to evolve antigenic variants via immunological pressure, and in humans and some other hosts positive selection of viruses occurs gradually in a process known as antigenic drift (3). The antigenic diversity of glycoproteins is used to further classify IAVs, of which there are 18 HA and 11 NA subtypes (4, 5). In addition, antigenically distinct isolates can also exist within the same subtype, referred to as drifted variants. IAVs exist mainly in the wild aquatic fowl reservoir (6-9) and only a small number of mammalian hosts are currently recognized as sustaining transmission of IAVs.

Canine influenza is a contagious respiratory disease of dogs caused by two IAVs: the H3N8 equine-origin influenza virus that transferred to dogs in the United States (US) around 1999 (10); and the avian virus-like H3N2 that transferred to dogs in Asia around 2005 (11). In 2015 an outbreak of H3N2 canine influenza virus (CIV) occurred in the US that was due to a virus similar to those detected in dogs in Asia (12). The H3N2 CIV has also been isolated from cats in a shelter in South Korea (13, 14). These CIVs represent new threats to canine health in the US and worldwide, as the virus may be spread through the racing track circuit, as was the case of the H3N8 strain (10, 15), while both viruses are spread widely within and among animal shelters and kennels (10, 16, 17).

93 CIVs are still relatively new viruses and because of the low levels of infection and
94 immunity among the broader population most dogs are susceptible to infection. Most
95 dogs infected by CIVs show only a mild respiratory illness, but severe outcomes are
96 also observed (18).

97 The recent emergence of CIVs (H3N8 and H3N2 CIVs) has increased the host
98 range of IAVs. The continuous circulation of CIVs in dog populations creates
99 opportunities for exposure of humans and other animals. As dogs are susceptible to
100 mammalian (equine-origin H3N8 CIV) and avian (avian-origin H3N2 CIV) IAVs, they
101 may have the potential to act as “mixing vessel” hosts for new IAV strains with potential
102 for human infection. Reassortments between H3N2 CIVs and human pandemic H1N1
103 IAV have been reported (19, 20), and the introduction of novel, antigenically distinct
104 glycoproteins (HA and NA) into the backbone of human IAVs have been associated with
105 human pandemics (21).

106 Vaccination is accepted as an effective strategy for the prevention of influenza
107 infections (22, 23). To date, three types of influenza virus vaccines have been approved
108 by the US Food and Drug Administration (FDA) for human use: recombinant viral HAs,
109 inactivated influenza vaccines (IIVs), and live attenuated influenza vaccines (LAIVs)
110 (24-26, 22, 27). In dogs, only IIV against both H3N8 and H3N2 CIVs are commercially
111 available. However, we have recently reported the generation of recombinant H3N8
112 CIVs containing truncated or a deleted non-structural 1 (NS1) protein as potential LAIVs
113 candidates for the treatment of H3N8 CIV infections (28).

114 IIVs are administered intramuscularly and elicit humoral immunity by inducing the
115 production of neutralizing antibodies that target epitopes on HA (29, 26). On the other

hand, LAIV more closely mimic the natural route of viral infection and elicit both cellular and humoral immune responses (24), providing better immunogenicity and protection (30, 31, 22).

In mammals IAV is a respiratory pathogen that replicates in the cooler (33°C) upper respiratory tract, in addition to replicating in the warmer (37°C) conditions of the lower respiratory tract (32). This temperature difference has allowed for the development of cold-adapted (ca), temperature-sensitive (ts), attenuated (att) viruses that replicate in the upper respiratory tract but do not damage the lower respiratory tract due to the elevated temperatures restricting replication (33). For human viruses these ca, ts, att properties have been mapped to five amino acid residues located in three viral proteins of A/Ann Arbor/6/60 H2N2 (A/AA/6/60): the polymerase basic 2 (PB2) N265S, the polymerase basic 1 (PB1) K391E, D581G, and A661T and the nucleoprotein (NP) D34G (34, 35). The mechanisms of attenuation are not fully understood but most likely involve multiple steps in the replication cycle of the virus (33). Importantly, when the ts signature of A/AA/6/60 was introduced into influenza A/Puerto Rico/8/34 H1N1 (PR8) or A/California/04/09 H1N1 (pH1N1) viruses, a similar ts phenotype was observed in tissue culture cells and in the mouse model of infection (36-38).

In order to develop a LAIV for the treatment of CIV H3N8 infections, we introduced the ts, ca, att mutations identified in the A/AA/6/60 LAIV into CIV H3N8 (referred to henceforth as LACIV H3N8) using reverse genetics (39). LACIV H3N8 replicated efficiently *in vitro* at 33ºC but not 37ºC or 39ºC. Compared to CIV H3N8 wild-type (WT), LACIV H3N8 was attenuated *ex vivo* and *in vivo* but was able to induce protective immunity in mice against H3N8 WT upon a single intranasal dose, demonstrating its

feasibility as a safe, immunogenic and protective LAIV candidate.

MATERIALS AND METHODS

Cells and viruses

Human embryonic kidney 293T cells (293T; ATCC CRL-11268) and Madin-Darby canine kidney cells (MDCK; ATCC CCL-34) were grown at 37ºC with 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 µg/ml; L-glutamine, 2 mM) (40).

Recombinant wild-type (WT) and live-attenuated (LACIV) H3N8 CIVs were generated using A/canine/NY/dog23/2009 H3N8 plasmid-based reverse genetics techniques (41) and grown in MDCK cells at 33ºC. Influenza A/Ca/IL/41915/2015 H3N2 was also grown in MDCK cells at 33ºC. For infections, virus stocks were diluted in phosphate buffered saline (PBS), 0.3% bovine albumin (BA) and 1% PS (PBS/BA/PS). After viral infections, cells were maintained in DMEM with 0.3% BA, 1% PSG, and 1 µg/ml TPCK-treated trypsin (Sigma) (39).

Plasmids

To generate H3N8 LACIV, the PB2 and PB1 genes were subcloned in a pUC19 plasmid (New England Biolabs) and then ts mutations (PB2 N265S; and PB1 K391E, D581G, and A661T) were introduced by site-directed mutagenesis. The presence of introduced mutations was confirmed by sequencing. Mutated PB2 and PB1 viral segments were subcloned from pUC19 into the ambisense pDZ plasmid for virus rescue. To test the ability of WT and LACIV H3N8 polymerases to replicate and transcribe at different temperatures (33ºC, 37ºC and 39ºC) using a minigenome assay, we engineered a pPolI plasmid containing the canine RNA polymerase I (Pol-I) promoter

and the mouse Pol-I terminator separated by SapI endonuclease restriction sites (cpPol-I). The canine Pol-I promoter was obtained by PCR from MDCK cells (42). Then, the Gaussia luciferase (Gluc) reporter gene containing the 3' and the 5' non-coding regions of the viral NP (v)RNA was cloned into the cpPol-I plasmid to generate the cpPol-I Gluc reporter plasmid. All plasmids were confirmed by sequencing (ACGT, Inc). Primers for the generation of the different plasmids are available upon request.

Minigenome assays

MDCK cells (12-well plate format, $5x10^5$ cells/well, triplicates) were co-transfected in suspension using Lipofectamine 2000 with 250 ng of each of the H3N8 WT or LACIV ambisense pDZ PB2, PB1, PA and NP plasmids, together with 500 ng of the cpPol-I Gluc plasmid. A mammalian expression pCAGGS plasmid encoding Cypridina luciferase (Cluc, 100 ng) was also included to normalize transfection efficiencies (43). Cells transfected in the absence of the pDZ NP plasmid were used as negative control. At 24 hours (h) post-transfection, Gluc and Cluc expression levels were determined using a Luciferase Assay kit (New England BioLabs) and quantified with a Lumicount luminometer (Packard). Fold induction over the level of induction for the negative control (absence of NP) was determined. The mean values and standard deviations (SDs) were calculated and statistical analysis was performed using a two-tailed Student *t* test using Microsoft Excel software.

Virus rescue

Virus rescues were performed as previously described (44, 40). Briefly, co-cultures (1:1) of 293T/MDCK cells (6-well plate format, 10^6 cells/well, triplicates) were co-transfected in suspension, using Lipofectamine 2000 (Invitrogen), with 1 µg of the eight-

ambisense H3N8 WT CIV (pDZ-PB2, -PB1, -PA, -HA, -NP, -NA, -M and –NS) plasmids. To rescue the H3N8 LACIV, WT PB2 and PB1 pDZ plasmids were substituted by those containing PB2 and PB1 H3N8 LACIV. At 12 h post-transfection, transfection medium was replaced with post-infection (p.i.) medium containing DMEM supplemented with 0.3% BSA, 1% PSG, and 0.5 µg/ml TPCK-treated trypsin (Sigma). Tissue culture supernatants (TCS) were collected 3 days post-transfection, clarified, and used to infect fresh monolayers of MDCK cells (6-well plate format, 10^6 cells/well, triplicates). At 3 days p.i., recombinant viruses were plaque purified and scaled up using MDCK cells at 33ºC (40). Virus stocks were titrated by standard plaque assay (plaque forming units, PFU/ml) in MDCK cells at 33ºC (40).

Virus growth kinetics

Multicycle growth analyses were performed by infecting confluent monolayers of MDCK cells (12-well plate format, $5x10^5$ cells/well, triplicates) at a multiplicity of infection (MOI) of 0.001. Viral titers in TCS collected at various times p.i. were determined by immunofocus assay (fluorescent forming units, FFU/ml) in MDCK cells as previously described (40). Briefly, confluent MDCK cells (96-well plate format, $5 x10^4$ cells/well, triplicates) were infected with 10-fold serial dilutions of H3N8 WT or LACIV. At 12 h p.i., cells were fixed and permeabilized (4% formaldehyde, 0.5% Triton X-100 in PBS) for 15 min at room temperature. After washing with PBS, cells were incubated in blocking solution (2.5% BSA in PBS) for 1 h at room temperature and then incubated with 1µg/ml of an anti-NP monoclonal antibody (HB-65, ATTC) for 1 h at 37ºC. After washing with PBS, cells were incubated with FITC-conjugated secondary anti-mouse antibody (Dako)

for 1 h at 37°C. The mean values and SDs were calculated using Microsoft Excel software.

Animal experiments

Adult (5- to 7-week-old) female WT C57BL/6 mice were purchased from the National Cancer Institute (NCI) and maintained in the animal care facility at the University of Rochester under specific pathogen-free conditions. Animal experiments were approved by the University Committee of Animal Resources and complied with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (45). Mice were anesthetized intraperitoneally (i.p.) with 2,2,2-tribromoethanol (Avertin; 240 mg/kg of body weight) and then inoculated intranasally (i.n.) with 30 µl of the indicated amounts of H3N8 WT or LACIV or H3N2 WT. Alternatively, 100 µl of a commercially available inactivated H3N8 CIV vaccine (Nobivac, Merck Animal Health) or inactivated H3N2 CIV vaccine (Zoetis) were inoculated intramuscularly (i.m). Virus replication was determined by measuring viral titers in the lungs of infected mice at the indicated days p.i. To that end, three mice in each group were euthanized by administration of a lethal dose of Avertin and exsanguination, and lungs were collected and homogenized. Virus titers were determined by immunofocus assay (FFU/ml) as indicated above. Mice sera were collected by submandibular bleeding 24 h prior to viral challenges and evaluated for the presence of influenza virus antibodies by enzyme-linked immunosorbent assays (ELISA) and neutralizing antibodies by hemagglutination inhibition (HAI) assays.

Evaluation of T cells response in lung and spleen

Mice (N=4) were immunized as describe above, with 10^3 PFU of H3N8 WT or LACIV,

253 mock vaccinated with PBS, or vaccinated with 100 µl of Nobivac (IIV).

254 ***Cellular Preparations:*** Ten days p.i., lungs and spleens were perfused with PBS,
255 removed, and separated into right and left lobes. Lung tissue was dissociated in C
256 tubes by the GentleMACS (Miltenyi Biotek) using the Lung01 program. Samples
257 were incubated in 5 mL [2µg/mL] Collagenase II in RPMI +8% FBS at 37°C for 30
258 minutes, with gentle agitation every 10 minutes. After digestion, samples were
259 further dissociated using the Heart01 program. Cell suspensions were filtered
260 through 70 µm filters prior to 75:40 Percoll (GE Healthcare) discontinuous gradient
261 separation. The top layer, containing fat and other debris, was removed by
262 aspiration. The cell layer was harvested and washed, prior to counting and staining.
263 Single-cell suspensions were prepared from collected spleens by disruption in RPMI
264 +8% FBS. Counting was achieved through Trypan blue exclusion on a hemocytometer.
265 ***Flow cytometry:*** Single cell suspensions were stained in PBS containing 1%
266 FBS, purified CD16/32 (clone 2.4G2), NP and PA tetramers (46), and the following
267 antibodies: TCRβ-PerCPCy5.5, CD8α-FITC, CD4-BV421, CD44-APCCy7, and CD62L-
268 PECy7. Cells were subsequently stained for viability using Live Dead Aqua (Invitrogen).
269 All antibodies were obtained from eBioscience, BD Biosciences, or Biolegend. PA and
270 NP tetramers were obtained from the NIH tetramer core facility (Atlanta, GA). Cells
271 were analyzed by an LSRII (BD Biosciences) in the University of Rochester Flow
272 Cytometry core facility and analyzed using FlowJo software (Tree Star).

273 **ELISAs**

274 ELISAs were performed as previously described (40) by coating 96-well plates at
275 4°C for 16 h with lysates from mock, H3N8 or H3N2 WT CIV-infected MDCK cells, or

with H3N2 CIV HA (250 ng per well; catalog number for IRR: FR-1478) or NA (250 ng per well; catalog number for IRR: FR-1479). After blocking with 1% BSA for 1 h at room temperature, plates were incubated with 2-fold serial dilutions (starting dilution of 1:50) of mice sera for 1 h at 37°C. After incubation, plates were washed with H_2O, and incubated with a HRP-conjugated goat anti-mouse IgG (1:2,000; Southern Biotech) for 1 h at 37°C. Reactions were then developed with tetramethylbenzidine (TMB) substrate (BioLegend) for 10 min at room temperature, quenched with 2N H_2SO_4, and read at 450 nm (Vmax kinetic microplate reader; Molecular Devices).

HAI assays

To evaluate the presence of H3N8 CIV neutralizing antibodies, mice sera were treated with receptor-destroying enzyme (RDE; Denka Seiken) and heat inactivated for 30 min at 56°C. Sera were then serially 2-fold diluted (starting dilution of 1:50) in 96-well V-bottom plates and mixed 1:1 with 4 hemagglutinating units (HAU) of WT H3N8 CIV for 30 min at room temperature. The HAI titers were determined by adding 0.5% turkey red blood cells (RBCs) to the virus-antibody mixtures for 30 min on ice, as previously described (40). The GMT and SDs from individual mice (N= 6) were calculated from the last well where hemagglutination was inhibited.

Canine tracheal explants preparation and virus titrations

Three dog tracheas were harvested from healthy Beagles (Charles River Laboratories) that had been used as negative controls in unrelated studies. Briefly, tracheas were collected aseptically immediately upon euthanasia and transported in pre-warmed medium as previously described (18). Tracheas were washed 5 times for a total period of 4h and maintained at 33°C, 5% CO_2, and 95% humidity between washes.

299 The connective tissue was removed and the trachea was then open lengthwise. Each
300 tracheal ring was divided in four 0.25 cm^2 explants and transferred with the epithelium
301 facing upwards onto an agarose plug covered by a sterile filter. The explants were kept
302 for a total of 6 days at 33°C, 5% CO_2, and 95% humidity.

303 Tracheal explants were infected after a period of 24 h post-preparation (designed as
304 day zero) with 200 PFU of WT or LACIV H3N8, or mock infected with culture medium.
305 Inoculated explants were sampled for virus quantification, bead clearance assays, and
306 histology at days 0, 1, 3 and 5 p.i. Viral replication was evaluated by plaque assays on
307 MDCK cells.

308 **Estimation of bead clearance time**

309 The ciliary function of tracheal explants was evaluated as previously described (18),
310 by placing five microliters of polystyrene microsphere beads (Polysciences,
311 Northampton, UK) on the explants apical surface and measuring the time to displace
312 the beads.

313 **Histological analysis and immunohistochemistry**

314 After collection, the explants were fixed in 10% buffered formalin for a minimum of
315 48 h, before paraffin embedding and sectioning. Subsequently, 4 μm sections of paraffin
316 embedded tissue were either stained with haematoxylin and eosin (H&E) for
317 histopathological evaluation or immunostained for the viral NP using standard
318 procedures as previously described (18). For NP immunostaining, the Dako supervision
319 system was used following the manufacturer's instructions, along with a monoclonal

mouse anti-NP (clone HB65; dilution 1:500). Slides were counterstained with Mayer's hematoxylin. Histological images were captured with cellD software (Olympus).

RESULTS

Generation and characterization of H3N8 LACIV

We introduced four ts mutations identified in previous studies into the PB2 and PB1 genes of H3N8 CIV (34, 47) (**Fig. 1**). No mutation was introduced into the viral NP since H3N8 CIV NP already contains a G at position 34.

To determine whether the mutations introduced into the PB2 and PB1 genes confer a ts phenotype to the H3N8 CIV polymerase, we performed a minigenome assay. Both, WT and LACIV H3N8 resulted in similar Gluc expression levels at 33ºC (**Fig. 1B**), but Gluc expression was reduced at higher temperatures (37ºC and 39ºC) in cells transfected with the H3N8 LACIV plasmids. This shows that those mutations resulted in a ts phenotype when introduced in the H3N8 CIV, as previously described for A/AA/6/60 H2N2 and other influenza viruses (36-38).

We next generated an H3N8 LACIV using plasmid-based reverse genetic approaches (40, 48) and evaluated the viral replication kinetics in MDCK cells infected at low (0.001) MOI, and compared to the WT H3N8 CIV (**Fig. 2**). At 33ºC, both WT and LACIV H3N8 grew with the same kinetics and reached similar high titers (10^7 FFU/ml) at 48-72 h p.i. (**Fig. 2A**). At higher temperatures (37ºC and 39ºC) the WT H3N8 CIV replicated at similar levels as those observed at 33ºC while H3N8 LACIV titers were reduced ~2-3-logs at 37ºC (**Fig. 2B**) or was not detected at 39ºC (**Fig. 2C**). These results demonstrate that mutations introduced in PB2 and PB1 conferred a ts phenotype to H3N8 CIV.

LACIV H3N8 is attenuated *in vivo* in mice

As the H3N8 LACIV presented defects in replication at higher (37ºC and 39ºC) temperatures, we next investigated whether H3N8 LACIV was also attenuated in mice. No signs of infection were detected after infection with WT H3N8 CIV. Therefore, CIV titers in the lungs of infected (10^5 PFU) mice were determined on days 2 (N=3) and 4 (N=3) p.i. and used as a measure of viral attenuation (**Fig. 3**). Notably, virus titers in the lungs were only detected in mice inoculated with WT H3N8 CIV and no virus was detected in mice infected with the H3N8 LACIV. These results indicate that H3N8 LACIV is also attenuated *in vivo*.

Intranasal vaccination with H3N8 LACIV induces protective immunity against WT H3N8 CIV challenge

To evaluate the immunity generated by the H3N8 LACIV, mice (N=6) were vaccinated (i.n.) with 10^3 PFU of H3N8 WT or LACIV, mock vaccinated with PBS, or vaccinated (i.m.) with 100 µl of Nobivac, a commercial IIV against H3N8 CIV (**Fig. 4**). Humoral immune responses were evaluated in mice sera collected 2 weeks later (**Fig. 4A**). Total H3N8 CIV antibody responses were characterized by ELISA using cell lysates from mock- or H3N8 CIV-infected MDCK cells (48). Mice vaccinated with the H3N8 LACIV elicited high serum IgG titers against parental H3N8 CIV, while antibody titers of mice vaccinated with Nobivac were lower than those in the H3N8 LACIV or WT vaccinated mice (**Fig. 4A**). Additionally, HAI assays were performed to examine the presence of anti-HA neutralizing antibodies on sera from vaccinated mice (**Fig. 4B**), showing that HAI titers against CIV H3N8 were higher in mice vaccinated with the H3N8 LACIV than those observed in mice vaccinated with the H3N8 IIV.

To further examine the immunogenicity of LACIV, we evaluated if the virus can induce a localized CD8 T cell response (**Fig. 5**). To this end, mice (N=4) were

388 immunized as described above and ten days p.i., lungs and spleen samples were
389 collected and single-cell preparations made for flow cytometric analysis (49). Live, CD8
390 T cells were further gated for a H3N8 CIV-specific population (49, 46). Results showed
391 that vaccination with H3N8 LACIV induced elevated levels of NP and PA-specific lung
392 CD8 T cells, similar to those induced by WT virus in both lungs (**Fig. 5A**) and spleen
393 (**Fig. 5B**). Importantly, animals vaccinated with Nobivac did not show a T cell response
394 either in the lung (**Fig. 5A**) or the spleen (**Fig. 5B**), highlighting important differences in
395 induced immunity between H3N8 LACIV and the H3N8 IIV.
396 We next evaluated the ability of H3N8 LACIV to induce protective immunity. Mice
397 (N=6) were vaccinated i.n. with 10^3 PFU of H3N8 WT or LACIV, i.m. with 100 µl of the
398 IIV Nobivac, or mock vaccinated with PBS. Two weeks later mice were challenged with
399 10^5 PFU of WT H3N8 CIV and viral titers in the lungs of infected mice (N=3/group) were
400 determined 2 and 4 days after challenge (**Fig. 6**). Mock-vaccinated mice showed high
401 viral titers in the lungs at days 2 and 4 p.i., while mice immunized with H3N8 WT CIV
402 and with LACIV showed no detectable virus in the lungs at those times (**Fig. 6**). Mice
403 vaccinated with the H3N8 IIV showed high viral titers at day 2, but no detectable virus at
404 day 4 p.i. (**Fig. 6**).
405 **H3N8 LACIV is attenuated in canine tracheal explants as compared to H3N8**
406 **WT CIV**
407 To compare LACIV and WT H3N8 CIV pathogenicity and replication efficiency at the
408 site of infection within dogs (**Fig. 7**), we inoculated dog tracheal explants with each virus
409 and compared histological lesions (**Fig. 7A**), viral NP expression (**Fig. 7B**), changes in
410 ciliary function (**Fig. 7C**) and viral replication (**Fig. 7D**) at different times (days 1, 3 and

411 5) p.i. The H3N8 WT CIV induced major histological changes in dog tracheal explants,
412 with thinning and desquamation of the epithelium, loss of cilia (**Fig 5A**), and significant
413 reduction of ciliary function (**Fig. 7C**) between days 1 and 5 p.i. Histological damages
414 induced by H3N8 LACIV were delayed and reduced compared to WT H3N8 CIV, as the
415 epithelium maintained its normal thickness until day 3 p.i. (**Fig. 7A**) and the ciliary
416 function (**Fig. 7C**) was only significantly reduced from day 3 p.i. Viral kinetics (**Fig. 7D**)
417 and NP expression (**Fig. 7B**) were comparable between the two viruses, although only
418 WT H3N8 CIV was detectable at day 1 p.i. (**Fig. 7D**). Overall, these results indicate that
419 H3N8 LACIV pathogenicity is attenuated in canine tracheal explants compared to its WT
420 counterpart.

421 **H3N8 LACIV provides limited protection against heterologous H3N2 CIV**
422 **challenge**

423 We next evaluated if H3N8 LACIV could induce protective immunity against a
424 heterologous H3N2 CIV challenge (**Fig. 8**). Mice (N=6) were vaccinated (i.n.) with 10^3
425 PFU of WT or LACIV H3N8, mock vaccinated with PBS, or vaccinated (i.m.) with 100 µl
426 of the H3N8 IIV Nobivac or a commercial H3N2 IIV (Zoetis). Antibodies against H3N2
427 CIV were evaluated by ELISA using cell lysates from mock- or H3N2 CIV-infected
428 MDCK cells as antigens (**Fig. 8A**). In addition, antibodies against the HA (**Fig. 8B**) or
429 NA (**Fig. 8C**) proteins of H3N2 CIV were also evaluated. When the cell lysate was used
430 as antigen, antibodies against H3N2 CIV were detected in sera of mice vaccinated with
431 WT H3N8 CIV and, to a lower extent, in mice vaccinated with H3N8 LACIV, although
432 the levels were lower than those against H3N8 CIV (**Fig. 8A**). Similarly, using the
433 recombinant proteins (HA or NA) as antigens to perform the ELISA, only antibodies

against H3N2 CIV HA were detected in samples of animals immunized with WT H3N8 CIV (**Fig. 8B**). However no antibodies were detected against the NA protein (**Fig. 8C**). No detectable IgG antibodies against H3N2 CIV were detected in mice vaccinated with the H3N8 IIV Nobivac either using the cell extracts (**Fig. 8A**) or the recombinant proteins (**Figs. 8B and C**). The H3N2 CIV IIV induced higher IgG antibodies against H3N2 CIV in all cases, as expected (**Figs. 8A-8C**).

The lower level of cross-reactive immunity against H3N2 CIV upon vaccination with the H3N8 LACIV was confirmed after challenge (i.n.) with H3N2 CIV 2 weeks post-vaccination (**Fig. 8D**). Mock-vaccinated mice showed high H3N2 CIV titers that were undistinguishable from those seen in animals vaccinated with the H3N8 CIV IIV Nobivac. In contrast, mice vaccinated with the H3N2 CIV IIV showed reduced or undetectable titers at day 2 and 4 post-challenge, respectively. Although we observed similar H3N2 CIV titers at day 2 post-challenge, viral titers at day 4 p.i. in mice vaccinated with the H3N8 LACIV were ~100 times lower than those obtained in the mock vaccinated group. These results suggest that H3N8 LACIV can induce limited cross-reactive immune responses and heterologous protection, most probably mediated by a T cell response, against H3N2 CIV, but that the efficacy is lower than that obtained with the H3N2 IIV.

DISCUSSION

Here we report a novel LAIV prepared using plasmid-based reverse genetics techniques, which may be used for the prevention of H3N8 CIV. We have generated a recombinant H3N8 CIV containing the mutations responsible for the ts phenotype of the human A/AA/6/60 H2N2 LAIV, resulting in ts H3N8 CIV (LACIV) that was highly attenuated *in vivo* and *ex vivo* when compared to its WT counterpart. Our H3N8 LACIV was able to confer, upon a single i.n. immunization in mice, complete protection against challenge with WT H3N8 CIV. This demonstrates the feasibility of using the ts H3N8 LACIV as a safe, immunogenic and protective vaccine to control H3N8 CIV in dogs. However, the H3N8 LACIV showed limited immunogenicity and protection efficacy against the heterologous H3N2 CIV, suggesting the need of a different LACIV for the treatment and control of H3N2 CIV.

The ts, ca, att A/AA/6/60 H2N2 LAIV has been licensed for human use since 2003, and is used as a master donor virus (MDV) for the generation of both seasonal or potentially pandemic human LAIV by creating reassortant viruses containing the six H2N2-derived internal viral RNA (vRNA) segments (PB2, PB1, PA, NP, M, and NS) and the two glycoprotein-encoding vRNAs (HA and NA) from a virus that antigenically matches the strains predicted to circulate in the upcoming influenza season (seasonal vaccine) or potentially pandemic strains (pandemic vaccine) (50, 51). Five mutations (PB2 N265S; PB1 K391E, D581G, A661T; and NP D34G) are responsible for the ts phenotype of the A/AA/6/60 H2N2 LAIV, and those mutations also impart a strong ts phenotype and attenuation to other viral strains, such as PR8 (52, 37) and pH1N1 (38).

Intranasal immunization is a desirable delivery method for providing optimal immunity to IAV because it leads to the generation of a mucosal immune responses, creating an immune barrier at the site of potential infection (53), as well as systemic humoral responses, cellular immunity (54-56, 51, 57). Similar to infection with WT IAV, LAIV immunization also leads to recruitment of influenza-specific CD8 T cells to the lungs (49, 58, 56, 59, 60), which provides immunity against heterologous influenza challenge (49, 58). Thus, a LAIV rather than an IIV is desired for the control of IAV infections (61, 22, 27, 23).

Since the emergence of H3N8 CIV in 1999 in the US and the H3N2 CIV in Asia (2005) and the US (2015), CIVs have been maintained mainly in animal shelters and kennels as those populations allow ready transmission of the virus (10, 62, 16, 17, 63). Strains of the H3 subtype of IAV infect a number of mammalian hosts, including humans, pigs, horses, dogs, cats, seals, as well as poultry (64-66, 6, 67, 63, 11). Naturally occurring H3N1 virus carrying the HA gene of an avian-like H3N2 CIV and the other seven segments of the human pH1N1 has been reported in dogs in Korea (20), suggesting that dogs could act as an intermediate host for genetic reassortment of IAV, including those that might infect humans. However, no transmission of H3N8 or H3N2 CIV transmission from dogs to humans has been reported to date. It may be possible to eradicate both H3N8 and H3N2 CIVs from the dog population through infection control as well as vaccination approaches. CIV LAIVs represent a better option for efficient CIV control and probably eradication since they induce better and faster antiviral immunity.

The H3N8 LACIV generated here was ts and attenuated in mice, induced protective immune responses against challenge with homologous H3N8 CIV WT, and the

502 responses were stronger than those obtained with a commercial H3N8 CIV IIV. Its
503 replication and pathogenesis were also restricted in canine tracheal explants, and we
504 are currently evaluating the safety, immunogenicity and protection efficacy of our H3N8
505 LACIV in dogs, the real target population.

506 To achieve protection in dogs, animals are vaccinated (i.m.) with 1 ml of the CIV
507 H3N8 IIV (Nobivac) using a two-dose regime (68). The average weight of a male or
508 female mouse is ~ 20 gr while the average weight of a female or male beagle dog is ~
509 10 kg. Thus, in principle mice should be vaccinated with 500 times less (just 2 µl) of the
510 CIV H3N8 IIV than dogs. However, in our experiments, mice were immunized (i.m.) with
511 100 µl of the CIV H3N8 IIV – a 50X higher dose than on a weight basis. In addition, to
512 evaluate the amount of antigen in the CIV H3N8 IIV, we performed an hemagglutination
513 assay (HA) using the commercial CIV H3N8 IIV or our CIV H3N8 LAIV. The assay
514 showed that mice vaccinated with the CIV H3N8 IIV were inoculated with approximately
515 10^6 viral particles/mouse of inactivated virus, 1,000 times more than the dose (10^3) of
516 the H3N8 CIV LAIV. The LAIV still elicited better antibody responses and protection as
517 compared to the CIV H3N8 IIV.

518 Segment eight of IAV encodes the NS1 viral protein, which controls the adaptive
519 immune responses by inhibiting the interferon (IFN)-antiviral response of the host (69).
520 Therefore, NS1 is a virulence factor that offers an attractive target for the development
521 of attenuated viruses as LAIVs. In fact, IAVs harboring a truncated-NS1 have been
522 shown as promising vaccines candidates (70, 71, 28, 72-77). In a recent work, we have
523 generated recombinant H3N8 CIVs containing truncated (NS1-126, NS1-99 or NS1-73)
524 or deleted (ΔNS1) NS1 proteins and tested them as potential LAIVs against CIV H3N8

infections (28). The recombinant NS1 mutant H3N8 CIVs were attenuated *in vivo* (mice) and *in vitro* (dog tracheal explants), but were able to confer complete protection against challenge with WT CIV H3N8 (28). Moreover, the immunogenicity and protection efficacy of our NS1 mutant H3N8 CIVs was also better than that observed with an H3N8 CIV IIV (28). Future research should determine which one of these attenuation strategies (NS1 mutant or ts H3N8 CIVs) is more efficient for their implementation as a LAIV for the prevention and control of H3N8 CIV in dogs.

ACKNOWLEDGMENTS

This research was partially funded by the University of Rochester Technology Development Fund to LM-S and CP. CC was supported by the Horserace Betting Levy Board and PRM was supported by the Medical Research Council of the United Kingdom (Grant MC_UU_120/14/9).

571 **FIGURE LEGENDS**

572 **Figure 1. Effect of temperature on the polymerase activity of H3N8 LACIV. A)**
573 **Schematic representation of segments 1 (PB2) and 2 (PB1) of WT (black) and**
574 **LACIV (gray) H3N8 CIV:** Amino acid substitutions N265S (PB2) and K391E, E581G,
575 and A661T (PB1) to generate the H3N8 LACIV are indicated. **B) Minigenome activity:**
576 MDCK cells (12 well plate format, $3x10^5$ cells/well, triplicates) were transiently co-
577 transfected with 250 ng of ambisense pDZ expression plasmids encoding the minimal
578 requirements for viral genome replication and gene transcription (PB2, PB1, PA and
579 NP), together with 500 ng of a vRNA-like expression plasmid encoding Gaussia
580 luciferase (Gluc) under the control of the canine polymerase I promoter (cpPol-I Gluc),
581 and 100 ng of a pCAGGS Cypridina luciferase (Cluc) plasmid to normalize transfection
582 efficiencies. After transfection, cells were placed at 33ºC, 37ºC or 39ºC and viral
583 replication and transcription was evaluated 24 h later by luminescence (Gluc). Gluc
584 activity was normalized to that of Cluc. Data represent means and SDs. Normalized
585 reporter expression is relative to that in the absence of pDZ NP plasmid. Data were
586 represented as relative activity considering WT H3N8 polymerase activity at each
587 temperature as 100%. *, $P<0.05$ using a Student's t test.

588 **Figure 2. Characterization of H3N8 LACIV *in vitro*:** MDCK cells (12 well plate format,
589 $3x10^5$ cells/well, triplicates) were infected (MOI of 0.001) with WT (black diamonds) and
590 LACIV (gray squares) H3N8 CIVs and incubated at 33ºC (**A**), 37ºC (**B**) and 39ºC (**C**).
591 TCS were collected at 12, 24, 48, 72, and 96 h p.i. and viral titers were determined by
592 immunofocus assay (FFU/ml). Data represent the means and SDs of the results

593 determined in triplicate. Dotted lines indicate the limit of detection (200 FFU/ml). *,
594 P<0.05 using a Student's t test.

595 **Figure 3. Attenuation of H3N8 LACIV *in vivo*:** Female 5- to-7-week-old C57BL/6 WT
596 mice (N=6) were infected (i.n.) with $1x10^5$ PFU of WT or LACIV H3N8 CIVs. Three mice
597 were sacrificed at days 2 (black) and 4 (gray) p.i. and lungs were harvested for virus
598 titrations using an immunofocus assay (FFU/ml). Data represent the means and SDs.
599 Dotted line indicate limit of detection (200 FFU/ml). ND, virus not detected.

600 **Figure 4. Immunogenicity of H3N8 LACIV:** Female 5- to-7-week-old C57BL/6 WT
601 mice (N=6) were vaccinated (i.n.) with $1x10^3$ PFU of WT or LACIV H3N8 CIVs. Mice
602 mock (PBS) vaccinated or vaccinated (i.m.) with 100 µl of an H3N8 CIV IIV (Nobivac)
603 were used as internal controls. **A**) **Induction of humoral responses:** At 14 days post-
604 vaccination, mice were bled and sera was evaluated for the presence of total IgG
605 antibodies against H3N8 CIV proteins using cell extracts of MDCK-infected cells by
606 ELISA. MDCK mock-infected cell extracts were used to evaluate the specificity of the
607 antibody response. OD, optical density. Data represent the means +/- SDs of the results
608 for 6 individual mice. * (Nobivac vs LACIV), ** (WT vs LACIV) or *** (WT vs Nobivac),
609 P<0.05 using a Student's t test. **B**) **Hemagglutination inhibition (HAI) titers:** Sera
610 from immunized mice were evaluated by HAI using four HAU of WT H3N8 CIV and 2-
611 fold serial dilutions of the indicated sera. ND, not detected. * (WT vs LACIV or Nobivac,
612 and LACIV vs Nobivac), P<0.05 using a Student's t test.

613 **Figure 5. CD8 T cell response induced by H3N8 LACIV:** Female 5- to-7-week-old
614 C57BL/6 WT mice (N=4) were vaccinated (i.n.) with $1x10^3$ PFU of WT or LACIV H3N8
615 CIVs. Mice mock (PBS) vaccinated or vaccinated (i.m.) with 100 µl of an H3N8 CIV IIV

(Nobivac) were used as internal controls. Ten days p.i., lungs (**A**) and spleen (**B**) were extracted and cells were prepared for flow cytometry. Live CD8 T cells specific for NP or PA tetramers were counted. Data represent the means +/- SDs of the results for 4 individual mice. * (WT vs LACIV, Nobivac or PBS and LACIV vs Nobivac or PBS), P<0.05 using a Student's t test.

Figure 6. Protection efficacy of H3N8 LACIV against homologous viral challenge: Female 5- to-7-week-old C57BL/6 WT mice (N=6) were vaccinated (i.n.) with $1x10^3$ PFU of WT or LACIV H3N8 CIVs. Mice mock (PBS) vaccinated or vaccinated (i.m.) with 100 µl of an H3N8 CIV IIV (Nobivac) were used as internal controls. At 15 days post-vaccination, mice were challenged (i.n.) with $1x10^5$ PFU of WT H3N8 CIV. To evaluate viral replication, mice were euthanized at days 2 (N=3, black) and 4 (N=3, gray) post-challenge and lungs were harvested, homogenized, and used to quantify viral titers by immunofocus assay (FFU/ml). The dotted line indicates the limit of detection (200 FFU/ml). ND, virus not detected. Data represent the means +/- SDs. *, P<0.05 using a Student's t test.

Figure 7. Comparison of WT and LACIV H3N8 CIV infection phenotypes in canine tracheal explants: A) Histological features of dog tracheal explants infected with 200 PFU of H3N8 WT CIV, H3N8 LACIV or mock-infected with infection media. Lesions are shown in sections stained with haematoxylin and eosin (H&E). **B**) CIV H3N8 infected cells were detected by immunostaining for the viral NP and positive cells are stained in brown. For both (**A**) and (**B**) panels, pictures are representatives of three independent experiments and black horizontal bars represent 20µm. **C**) Graphical representation of the bead clearance average time of CIV- or mock-infected dog tracheal explants for

three independent experiments. Data represent the means +/- SDs. Ns, p>0.05 (D1: LACIV vs Mock); *, p<0.05 (D1: WT vs Mock); **, p<0.01 (D3: LACIV vs Mock); ***, p<0.001 (D3: WT vs Mock); ****, p<0.0001 (D5: LACIV and WT vs Mock). **D**) Average viral replication of H3N8 LACIV and H3N8 WT CIV in canine tracheal explants from three independent experiments. Data represent the means +/- SDs. Dotted line indicate limit of detection (20 FFU/ml).

Figure.8: Immunogenicity and protection efficacy of H3N8 LACIV against heterologous H3N2 CIV challenge: Female 5- to-7-week-old C57BL/6 WT mice were vaccinated (i.n.) with $1x10^3$ PFU of WT and LACIV H3N8 CIVs. Mice mock (PBS) vaccinated or vaccinated (i.m.) with 100µl of the H3N8 (Nobivac) and an H3N2 CIV IIV (Zoetis) were used as internal controls. **A to C**) **Antibody cross-reactivity against the heterologous CIV H3N2:** At 14 days post-vaccination, mice were bled and sera was evaluated by ELISA for total IgG antibodies against H3N2 CIV proteins using cell extracts of MDCK-infected cells (**A**). Mock-infected MDCK cell extracts were used to evaluate the specificity of the antibody response. OD, optical density. Data represent the means +/- SDs of the results for 6 individual mice. * (Nobivac vs LACIV), ** (WT vs LACIV) or *** (WT vs Nobivac), P<0.05 using a Student's t test. Specific antibody response against recombinant HA (**B**) and NA (**C**) proteins from H3N2 CIV were evaluated by ELISA. Data represent the means +/- SDs of the results for pooled sera samples. * (WT vs LACIV), P<0.05 using a Student's t test. **D**) **Protection efficacy of H3N8 LACIV against heterologous H3N2 CIV challenge:** At 15 days post-vaccination, mice were challenged (i.n.) with $1x10^5$ PFU of WT H3N2 CIV. To evaluate WT H3N2 CIV replication, mice were sacrificed at days 2 (N=3, black) and 4 (N=3, gray) post-

challenge and lungs were harvested, homogenized, and used to evaluate the presence of virus by immunofocus assay (FFU/ml). The dotted line indicates the limit of detection (200 FFU/ml). ND, virus not detected. Data represent the means +/- SDs. *, P<0.05 using a Student's t test.

685 **REFERENCES**

686 1. **Baker, S. F., A. Nogales, and L. Martinez-Sobrido.** 2015. Downregulating viral
687 gene expression: codon usage bias manipulation for the generation of novel
688 influenza A virus vaccines. Future Virol **10:**715-730.

689 2. **Varghese, J. N., J. L. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt, and P.**
690 **M. Colman.** 1992. The structure of the complex between influenza virus
691 neuraminidase and sialic acid, the viral receptor. Proteins **14:**327-332.

692 3. **Carrat, F., and A. Flahault.** 2007. Influenza vaccine: the challenge of antigenic
693 drift. Vaccine **25:**6852-6862.

694 4. **Palese, P. S., ML.** 2007. Orthomyxoviridae: The Viruses and Their Replication.
695 In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A. (Eds.),
696 Fields Virology. 5th edition, Lippincott Williams and WIlkins.

697 5. **Tong, S., X. Zhu, Y. Li, M. Shi, J. Zhang, M. Bourgeois, H. Yang, X. Chen, S.**
698 **Recuenco, J. Gomez, L. M. Chen, A. Johnson, Y. Tao, C. Dreyfus, W. Yu, R.**
699 **McBride, P. J. Carney, A. T. Gilbert, J. Chang, Z. Guo, C. T. Davis, J. C.**
700 **Paulson, J. Stevens, C. E. Rupprecht, E. C. Holmes, I. A. Wilson, and R. O.**
701 **Donis.** 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog
702 **9:**e1003657.

703 6. **de Jong, J. C., D. J. Smith, A. S. Lapedes, I. Donatelli, L. Campitelli, G.**
704 **Barigazzi, K. Van Reeth, T. C. Jones, G. F. Rimmelzwaan, A. D. Osterhaus,**
705 **and R. A. Fouchier.** 2007. Antigenic and genetic evolution of swine influenza A
706 (H3N2) viruses in Europe. J Virol **81:**4315-4322.

7. **Taubenberger, J. K., and J. C. Kash.** 2010. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe **7:**440-451.

8. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-179.

9. **Yoon, S. W., R. J. Webby, and R. G. Webster.** 2014. Evolution and ecology of influenza a viruses. Curr Top Microbiol Immunol **385:**359-375.

10. **Crawford, P. C., E. J. Dubovi, W. L. Castleman, I. Stephenson, E. P. Gibbs, L. Chen, C. Smith, R. C. Hill, P. Ferro, J. Pompey, R. A. Bright, M. J. Medina, C. M. Johnson, C. W. Olsen, N. J. Cox, A. I. Klimov, J. M. Katz, and R. O. Donis.** 2005. Transmission of equine influenza virus to dogs. Science **310:**482-485.

11. **Song, D., B. Kang, C. Lee, K. Jung, G. Ha, D. Kang, S. Park, B. Park, and J. Oh.** 2008. Transmission of avian influenza virus (H3N2) to dogs. Emerg Infect Dis **14:**741-746.

12. 2015. Outbreak of canine influenza caused by new strain of virus. Javma-Journal of the American Veterinary Medical Association **246:**1049-1049.

13. **Jeoung, H. Y., S. I. Lim, B. H. Shin, J. A. Lim, J. Y. Song, D. S. Song, B. K. Kang, H. J. Moon, and D. J. An.** 2013. A novel canine influenza H3N2 virus isolated from cats in an animal shelter. Vet Microbiol **165:**281-286.

14. **Song, D. S., D. J. An, H. J. Moon, M. J. Yeom, H. Y. Jeong, W. S. Jeong, S. J. Park, H. K. Kim, S. Y. Han, J. S. Oh, B. K. Park, J. K. Kim, H. Poo, R. G. Webster, K. Jung, and B. K. Kang.** 2011. Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010. J Gen Virol **92:**2350-2355.

15. **Yoon, K. J., V. L. Cooper, K. J. Schwartz, K. M. Harmon, W. I. Kim, B. H. Janke, J. Strohbehn, D. Butts, and J. Troutman.** 2005. Influenza virus infection in racing greyhounds. Emerg Infect Dis **11:**1974-1976.

16. **Holt, D. E., M. R. Mover, and D. C. Brown.** 2010. Serologic prevalence of antibodies against canine influenza virus (H3N8) in dogs in a metropolitan animal shelter. J Am Vet Med Assoc **237:**71-73.

17. **Pecoraro, H. L., M. E. Spindel, S. Bennett, K. F. Lunn, and G. A. Landolt.** 2013. Evaluation of virus isolation, one-step real-time reverse transcription polymerase chain reaction assay, and two rapid influenza diagnostic tests for detecting canine Influenza A virus H3N8 shedding in dogs. J Vet Diagn Invest **25:**402-406.

18. **Gonzalez, G., J. F. Marshall, J. Morrell, D. Robb, J. W. McCauley, D. R. Perez, C. R. Parrish, and P. R. Murcia.** 2014. Infection and pathogenesis of canine, equine, and human influenza viruses in canine tracheas. J Virol **88:**9208-9219.

19. **Song, D., H. Kim, W. Na, M. Hong, S. J. Park, H. Moon, B. Kang, K. S. Lyoo, M. Yeom, D. G. Jeong, D. J. An, and J. K. Kim.** 2015. Canine susceptibility to human influenza viruses (A/pdm 09H1N1, A/H3N2 and B). J Gen Virol **96:**254-258.

20. **Song, D., H. J. Moon, D. J. An, H. Y. Jeoung, H. Kim, M. J. Yeom, M. Hong, J. H. Nam, S. J. Park, B. K. Park, J. S. Oh, M. Song, R. G. Webster, J. K. Kim, and B. K. Kang.** 2012. A novel reassortant canine H3N1 influenza virus between pandemic H1N1 and canine H3N2 influenza viruses in Korea. J Gen Virol **93:**551-554.

753 21. **Yen, H. L., and R. G. Webster.** 2009. Pandemic influenza as a current threat.
754 Curr Top Microbiol Immunol **333:**3-24.
755 22. **Pica, N., and P. Palese.** 2013. Toward a universal influenza virus vaccine:
756 prospects and challenges. Annu Rev Med **64:**189-202.
757 23. **Wong, S. S., and R. J. Webby.** 2013. Traditional and new influenza vaccines.
758 Clin Microbiol Rev **26:**476-492.
759 24. **Belshe, R. B., K. M. Edwards, T. Vesikari, S. V. Black, R. E. Walker, M.**
760 **Hultquist, G. Kemble, E. M. Connor, and C.-T. C. E. S. Group.** 2007. Live
761 attenuated versus inactivated influenza vaccine in infants and young children. N
762 Engl J Med **356:**685-696.
763 25. **Cox, M. M., P. A. Patriarca, and J. Treanor.** 2008. FluBlok, a recombinant
764 hemagglutinin influenza vaccine. Influenza Other Respir Viruses **2:**211-219.
765 26. **Osterholm, M. T., N. S. Kelley, A. Sommer, and E. A. Belongia.** 2012. Efficacy
766 and effectiveness of influenza vaccines: a systematic review and meta-analysis.
767 Lancet Infect Dis **12:**36-44.
768 27. **Pronker, E. S., E. Claassen, and A. D. Osterhaus.** 2012. Development of new
769 generation influenza vaccines: recipes for success? Vaccine **30:**7344-7347.
770 28. **Nogales, A., K. Huang, C. Chauche, M. L. DeDiego, P. R. Murcia, C. R.**
771 **Parrish, and L. Martinez-Sobrido.** 2017. Canine influenza viruses with modified
772 NS1 proteins for the development of live-attenuated vaccines. Virology **500:**1-10.
773 29. **Belongia, E. A., B. A. Kieke, J. G. Donahue, R. T. Greenlee, A. Balish, A.**
774 **Foust, S. Lindstrom, and D. K. Shay.** 2009. Effectiveness of inactivated

influenza vaccines varied substantially with antigenic match from the 2004-2005 season to the 2006-2007 season. J Infect Dis **199:**159-167.

30. **Gorse, G. J., R. B. Belshe, and N. J. Munn.** 1991. Superiority of live attenuated compared with inactivated influenza A virus vaccines in older, chronically ill adults. Chest **100:**977-984.

31. **Nogales, A., K. Huang, C. Chauche, M. L. DeDiego, P. R. Murcia, C. R. Parrish, and L. Martinez-Sobrido.** 2016. Canine influenza viruses with modified NS1 proteins for the development of live-attenuated vaccines. Virology **500:**1-10.

32. **Maassab, H. F.** 1968. Plaque formation of influenza virus at 25 degrees C. Nature **219:**645-646.

33. **Chan, W., H. Zhou, G. Kemble, and H. Jin.** 2008. The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature. Virology **380:**304-311.

34. **Cox, N. J., F. Kitame, A. P. Kendal, H. F. Maassab, and C. Naeve.** 1988. Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). Virology **167:**554-567.

35. **Snyder, M. H., R. F. Betts, D. DeBorde, E. L. Tierney, M. L. Clements, D. Herrington, S. D. Sears, R. Dolin, H. F. Maassab, and B. R. Murphy.** 1988. Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. J Virol **62:**488-495.

36. **Cox, A., S. F. Baker, A. Nogales, L. Martinez-Sobrido, and S. Dewhurst.** 2015. Development of a mouse-adapted live attenuated influenza virus that permits in

vivo analysis of enhancements to the safety of live attenuated influenza virus vaccine. J Virol **89:**3421-3426.

37. **Jin, H., H. Zhou, B. Lu, and G. Kemble.** 2004. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J Virol **78:**995-998.

38. **Zhou, B., Y. Li, S. D. Speer, A. Subba, X. Lin, and D. E. Wentworth.** 2012. Engineering temperature sensitive live attenuated influenza vaccines from emerging viruses. Vaccine **30:**3691-3702.

39. **Martinez-Sobrido, L., and A. Garcia-Sastre.** 2010. Generation of recombinant influenza virus from plasmid DNA. J Vis Exp.

40. **Nogales, A., S. F. Baker, E. Ortiz-Riano, S. Dewhurst, D. J. Topham, and L. Martinez-Sobrido.** 2014. Influenza A Virus Attenuation by Codon Deoptimization of the NS Gene for Vaccine Development. J Virol **88:**10525-10540.

41. **Feng, K. H., G. Gonzalez, L. Deng, H. Yu, V. L. Tse, L. Huang, K. Huang, B. R. Wasik, B. Zhou, D. E. Wentworth, E. C. Holmes, X. Chen, A. Varki, P. R. Murcia, and C. R. Parrish.** 2015. Equine and Canine Influenza H3N8 Viruses Show Minimal Biological Differences Despite Phylogenetic Divergence. J Virol **89:**6860-6873.

42. **Murakami, S., T. Horimoto, S. Yamada, S. Kakugawa, H. Goto, and Y. Kawaoka.** 2008. Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production. J Virol **82:**1605-1609.

43. **Cheng, B. Y., E. Ortiz-Riano, A. Nogales, J. C. de la Torre, and L. Martinez-Sobrido.** 2015. Development of live-attenuated arenavirus vaccines based on codon deoptimization. J Virol **89:**3523-3533.

44. **Nogales, A., S. F. Baker, and L. Martinez-Sobrido.** 2014. Replication-competent influenza A viruses expressing a red fluorescent protein. Virology **476C:**206-216.

45. **National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), and National Academies Press (U.S.).** 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, D.C.

46. **Reilly, E. C., K. Lambert-Emo, and D. J. Topham.** 2016. The Effects of Acute Neutrophil Depletion on Resolution of Acute Influenza Infection, Establishment of Tissue Resident Memory (TRM), and Heterosubtypic Immunity. PLoS One **11:**e0164247.

47. **Jin, H., B. Lu, H. Zhou, C. Ma, J. Zhao, C. F. Yang, G. Kemble, and H. Greenberg.** 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology **306:**18-24.

48. **Nogales, A., M. L. DeDiego, D. J. Topham, and L. Martinez-Sobrido.** 2016. Rearrangement of Influenza Virus Spliced Segments for the Development of Live-Attenuated Vaccines. J Virol **90:**6291-6302.

49. **Baker, S. F., H. Guo, R. A. Albrecht, A. Garcia-Sastre, D. J. Topham, and L. Martinez-Sobrido.** 2013. Protection against Lethal Influenza with a Viral Mimic. J Virol **87:**8591-8605.

50. **Maassab, H. F., and M. L. Bryant.** 1999. The development of live attenuated cold-adapted influenza virus vaccine for humans. Rev Med Virol **9:**237-244.

51. **Murphy, B. R., and K. Coelingh.** 2002. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. Viral Immunol **15:**295-323.

52. **Cox, A., S. F. Baker, A. Nogales, L. Martinez-Sobrido, and S. Dewhurst.** 2014. Development of a mouse-adapted, live-attenuated influenza virus that permits in vivo analysis of enhancements to the safety of LAIV. J Virol.

53. **Kohlmeier, J. E., and D. L. Woodland.** 2009. Immunity to respiratory viruses. Annu Rev Immunol **27:**61-82.

54. **Cheng, X., J. R. Zengel, A. L. Suguitan, Jr., Q. Xu, W. Wang, J. Lin, and H. Jin.** 2013. Evaluation of the humoral and cellular immune responses elicited by the live attenuated and inactivated influenza vaccines and their roles in heterologous protection in ferrets. J Infect Dis **208:**594-602.

55. **De Villiers, P. J., A. D. Steele, L. A. Hiemstra, R. Rappaport, A. J. Dunning, W. C. Gruber, and B. D. Forrest.** 2009. Efficacy and safety of a live attenuated influenza vaccine in adults 60 years of age and older. Vaccine **28:**228-234.

56. **Katsura, H., K. Iwatsuki-Horimoto, S. Fukuyama, S. Watanabe, S. Sakabe, Y. Hatta, S. Murakami, M. Shimojima, T. Horimoto, and Y. Kawaoka.** 2012. A

replication-incompetent virus possessing an uncleavable hemagglutinin as an influenza vaccine. Vaccine **30:**6027-6033.

57. **Victor, S. T., S. Watanabe, H. Katsura, M. Ozawa, and Y. Kawaoka.** 2012. A replication-incompetent PB2-knockout influenza A virus vaccine vector. J Virol.

58. **Guo, H., S. F. Baker, L. Martinez-Sobrido, and D. J. Topham.** 2014. Induction of CD8 T cell heterologous protection by a single dose of single-cycle infectious influenza virus. J Virol **88:**12006-12016.

59. **Powell, T. J., J. D. Silk, J. Sharps, E. Fodor, and A. R. Townsend.** 2012. Pseudotyped influenza A virus as a vaccine for the induction of heterotypic immunity. J Virol **86:**13397-13406.

60. **Uraki, R., M. Kiso, K. Iwatsuki-Horimoto, S. Fukuyama, E. Takashita, M. Ozawa, and Y. Kawaoka.** 2013. A novel bivalent vaccine based on a PB2-knockout influenza virus protects mice from pandemic H1N1 and highly pathogenic H5N1 virus challenges. J Virol **87:**7874-7881.

61. **Hoft, D. F., E. Babusis, S. Worku, C. T. Spencer, K. Lottenbach, S. M. Truscott, G. Abate, I. G. Sakala, K. M. Edwards, C. B. Creech, M. A. Gerber, D. I. Bernstein, F. Newman, I. Graham, E. L. Anderson, and R. B. Belshe.** 2011. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. J Infect Dis **204:**845-853.

62. **Hayward, J. J., E. J. Dubovi, J. M. Scarlett, S. Janeczko, E. C. Holmes, and C. R. Parrish.** 2010. Microevolution of canine influenza virus in shelters and its molecular epidemiology in the United States. J Virol **84:**12636-12645.

63. **Rivailler, P., I. A. Perry, Y. Jang, C. T. Davis, L. M. Chen, E. J. Dubovi, and R. O. Donis.** 2010. Evolution of canine and equine influenza (H3N8) viruses co-circulating between 2005 and 2008. Virology **408:**71-79.

64. **Bean, W. J., M. Schell, J. Katz, Y. Kawaoka, C. Naeve, O. Gorman, and R. G. Webster.** 1992. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J Virol **66:**1129-1138.

65. **Both, G. W., M. J. Sleigh, N. J. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol **48:**52-60.

66. **Bush, R. M., W. M. Fitch, C. A. Bender, and N. J. Cox.** 1999. Positive selection on the H3 hemagglutinin gene of human influenza virus A. Mol Biol Evol **16:**1457-1465.

67. **Epperson, S., M. Jhung, S. Richards, P. Quinlisk, L. Ball, M. Moll, R. Boulton, L. Haddy, M. Biggerstaff, L. Brammer, S. Trock, E. Burns, T. Gomez, K. K. Wong, J. Katz, S. Lindstrom, A. Klimov, J. S. Bresee, D. B. Jernigan, N. Cox, and L. Finelli.** 2013. Human infections with influenza A(H3N2) variant virus in the United States, 2011-2012. Clin Infect Dis **57 Suppl 1:**S4-S11.

68. **Larson, L. J., J. Henningson, P. Sharp, B. Thiel, M. S. Deshpande, T. Davis, H. Jayappa, T. Wasmoen, N. Lakshmanan, and R. D. Schultz.** 2011. Efficacy of the canine influenza virus H3N8 vaccine to decrease severity of clinical disease after cochallenge with canine influenza virus and Streptococcus equi subsp. zooepidemicus. Clin Vaccine Immunol **18:**559-564.

69. **Hale, B. G., R. E. Randall, J. Ortin, and D. Jackson.** 2008. The multifunctional NS1 protein of influenza A viruses. J Gen Virol **89:**2359-2376.

70. **Baskin, C. R., H. Bielefeldt-Ohmann, A. Garcia-Sastre, T. M. Tumpey, N. Van Hoeven, V. S. Carter, M. J. Thomas, S. Proll, A. Solorzano, R. Billharz, J. L. Fornek, S. Thomas, C. H. Chen, E. A. Clark, K. Murali-Krishna, and M. G. Katze.** 2007. Functional genomic and serological analysis of the protective immune response resulting from vaccination of macaques with an NS1-truncated influenza virus. J Virol **81:**11817-11827.

71. **Choi, E. H., M. S. Song, S. J. Park, P. N. Pascua, Y. H. Baek, H. I. Kwon, E. H. Kim, S. Kim, H. K. Jang, H. Poo, C. J. Kim, and Y. K. Choi.** 2015. Development of a dual-protective live attenuated vaccine against H5N1 and H9N2 avian influenza viruses by modifying the NS1 gene. Arch Virol **160:**1729-1740.

72. **Pica, N., R. A. Langlois, F. Krammer, I. Margine, and P. Palese.** 2012. NS1-truncated live attenuated virus vaccine provides robust protection to aged mice from viral challenge. J Virol **86:**10293-10301.

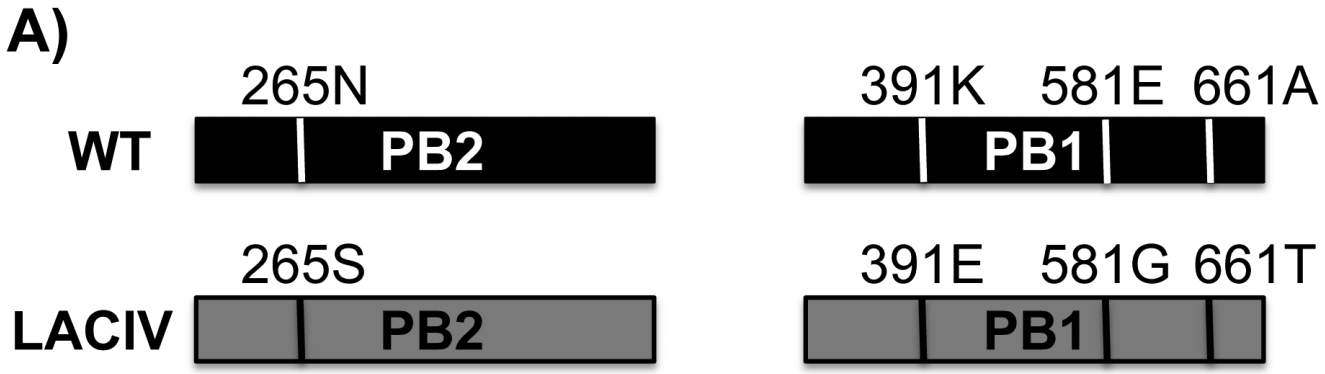
73. **Quinlivan, M., D. Zamarin, A. Garcia-Sastre, A. Cullinane, T. Chambers, and P. Palese.** 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol **79:**8431-8439.

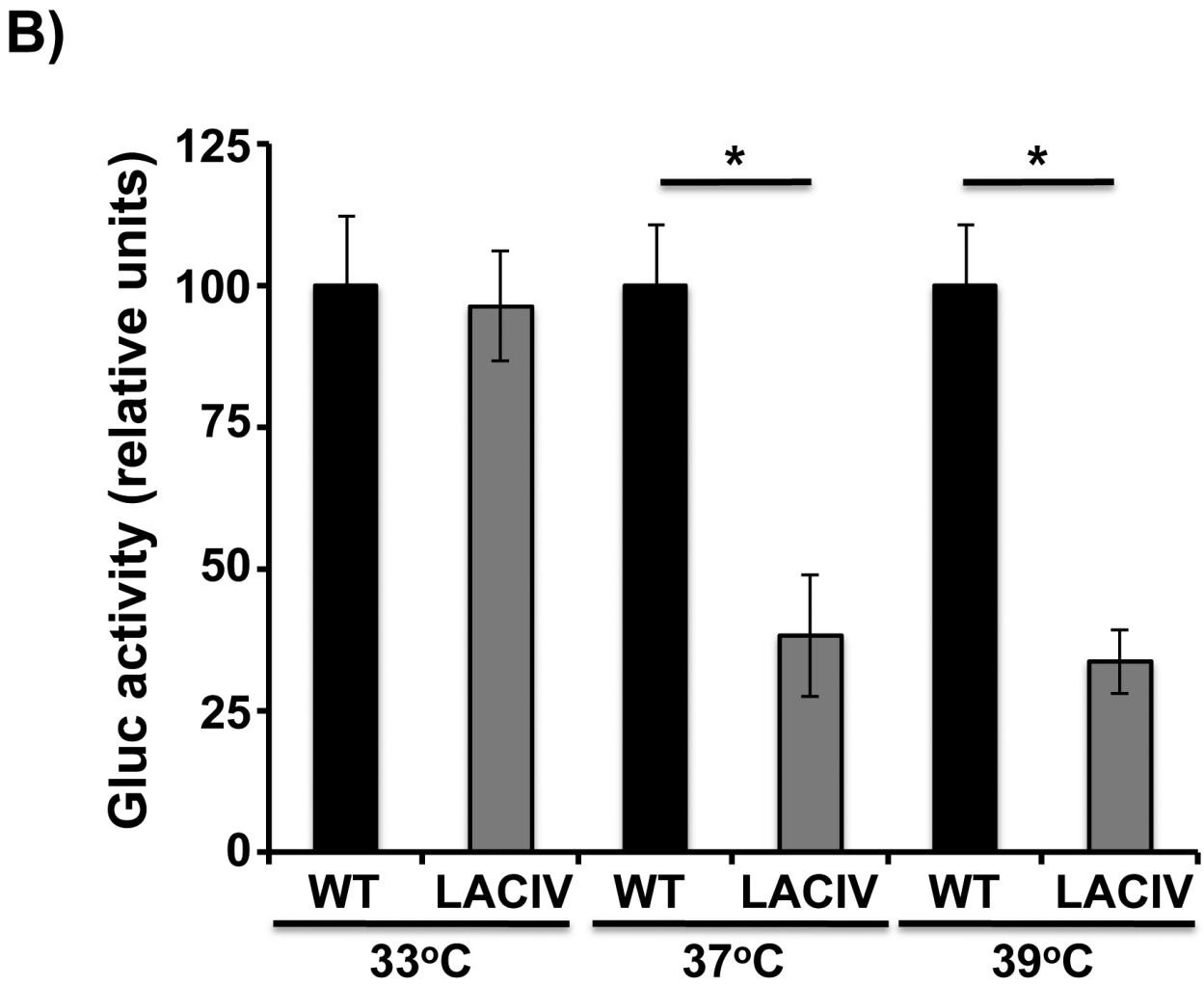
74. **Richt, J. A., P. Lekcharoensuk, K. M. Lager, A. L. Vincent, C. M. Loiacono, B. H. Janke, W. H. Wu, K. J. Yoon, R. J. Webby, A. Solorzano, and A. Garcia-Sastre.** 2006. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J Virol **80:**11009-11018.

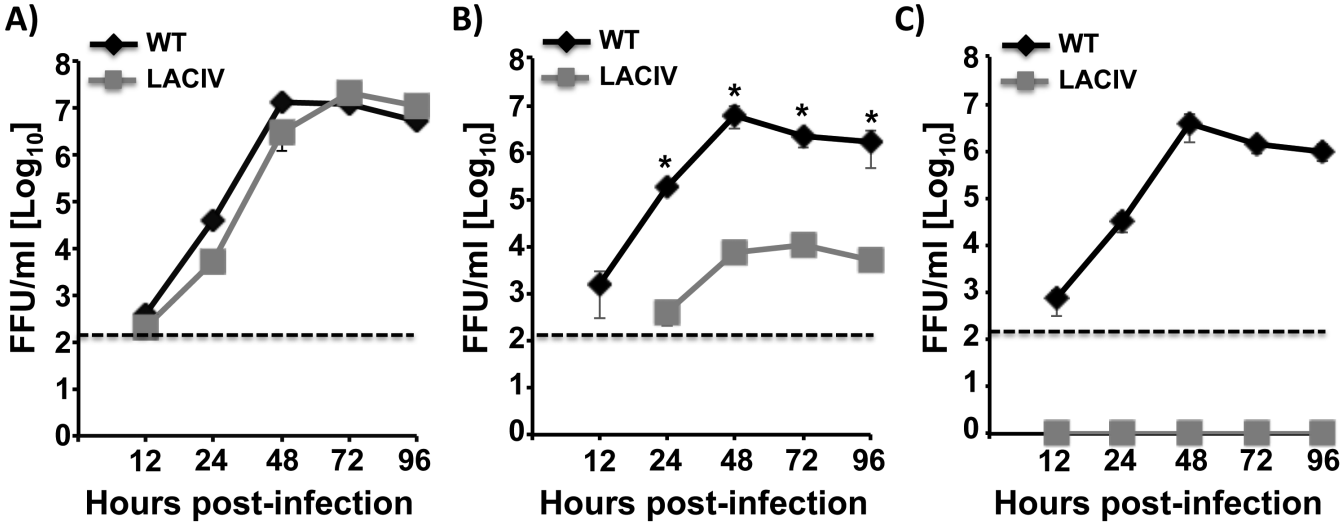
75. **Solorzano, A., R. J. Webby, K. M. Lager, B. H. Janke, A. Garcia-Sastre, and J. A. Richt.** 2005. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J Virol **79:**7535-7543.

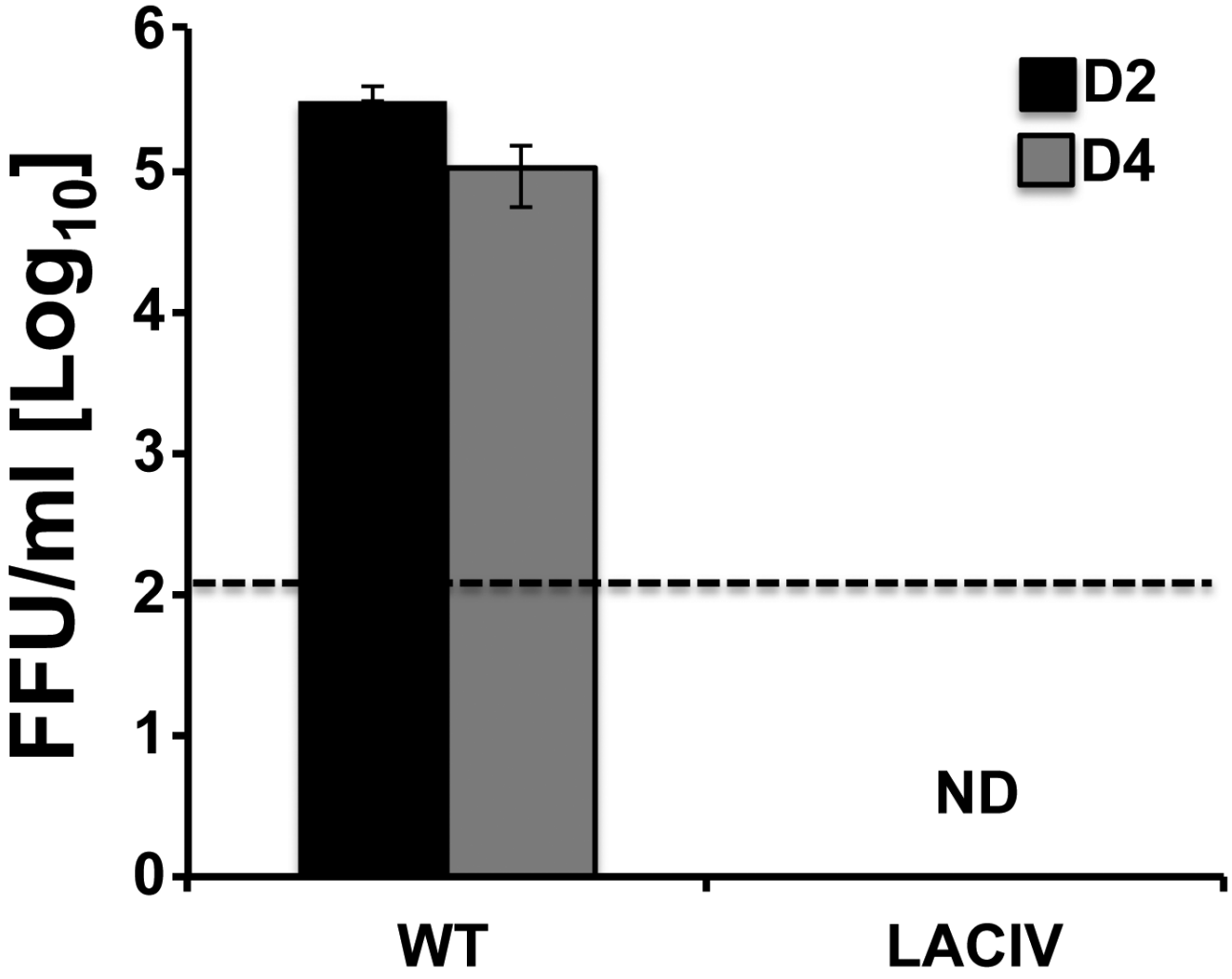
76. **Steel, J., A. C. Lowen, L. Pena, M. Angel, A. Solorzano, R. Albrecht, D. R. Perez, A. Garcia-Sastre, and P. Palese.** 2009. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J Virol **83:**1742-1753.

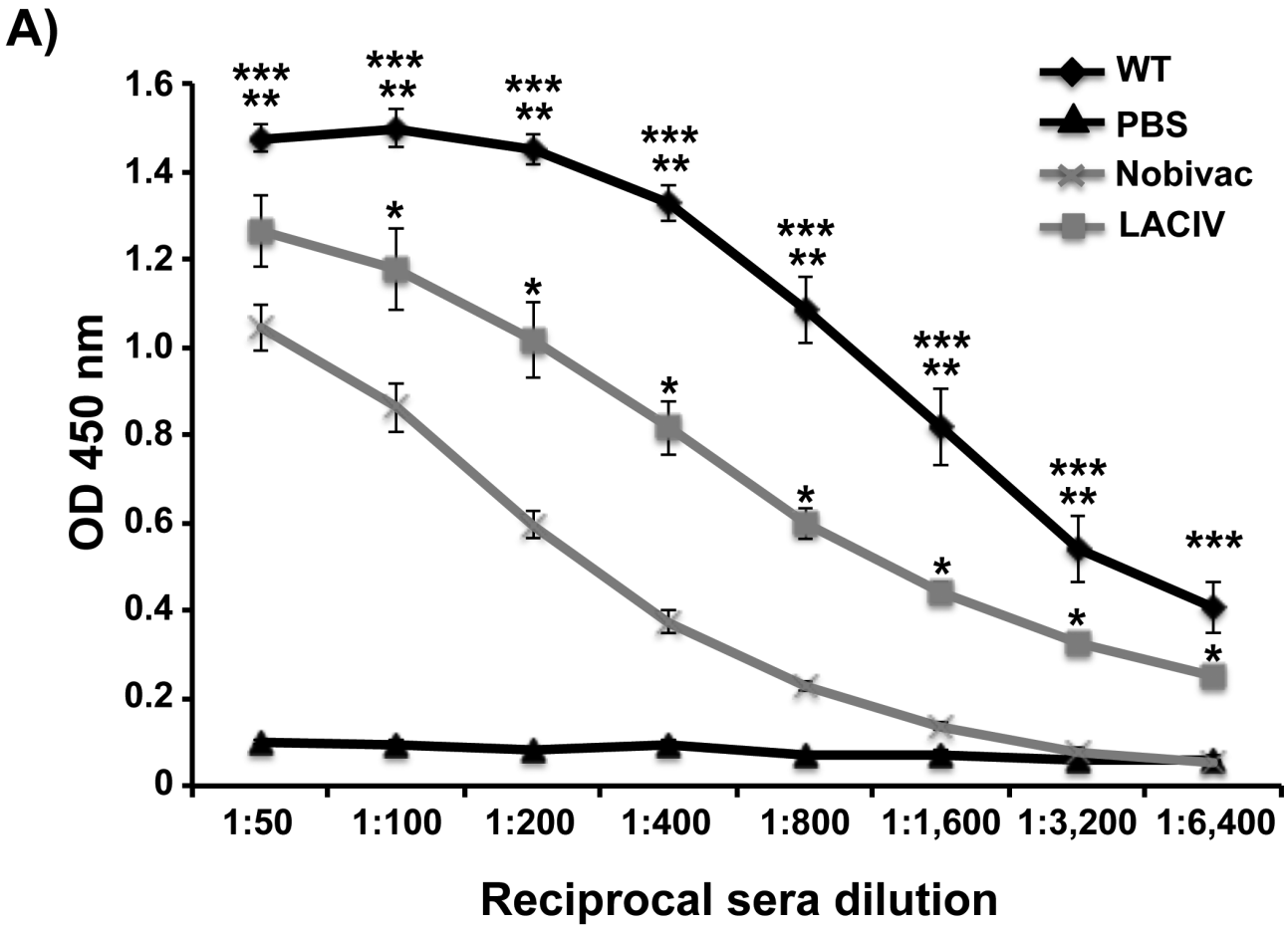
77. **Vincent, A. L., W. Ma, K. M. Lager, B. H. Janke, R. J. Webby, A. Garcia-Sastre, and J. A. Richt.** 2007. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. Vaccine **25:**7999-8009.

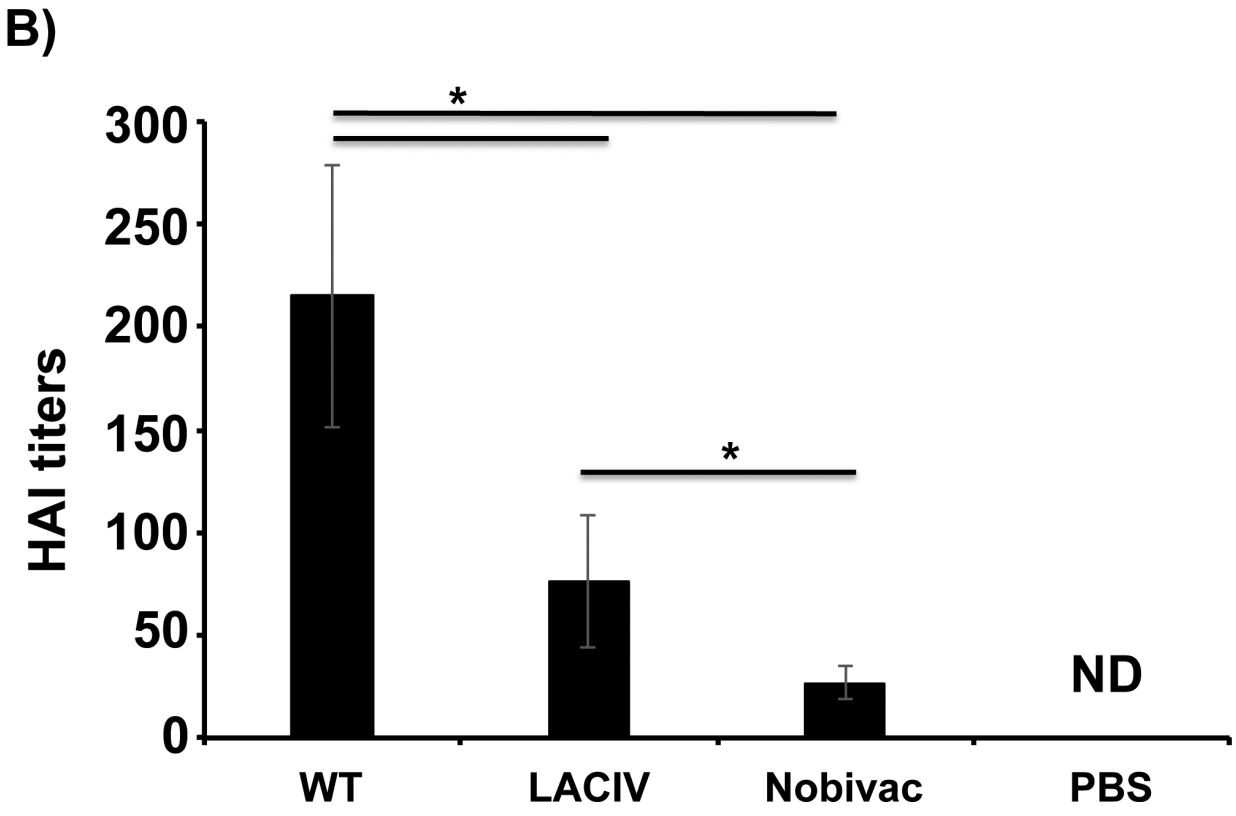


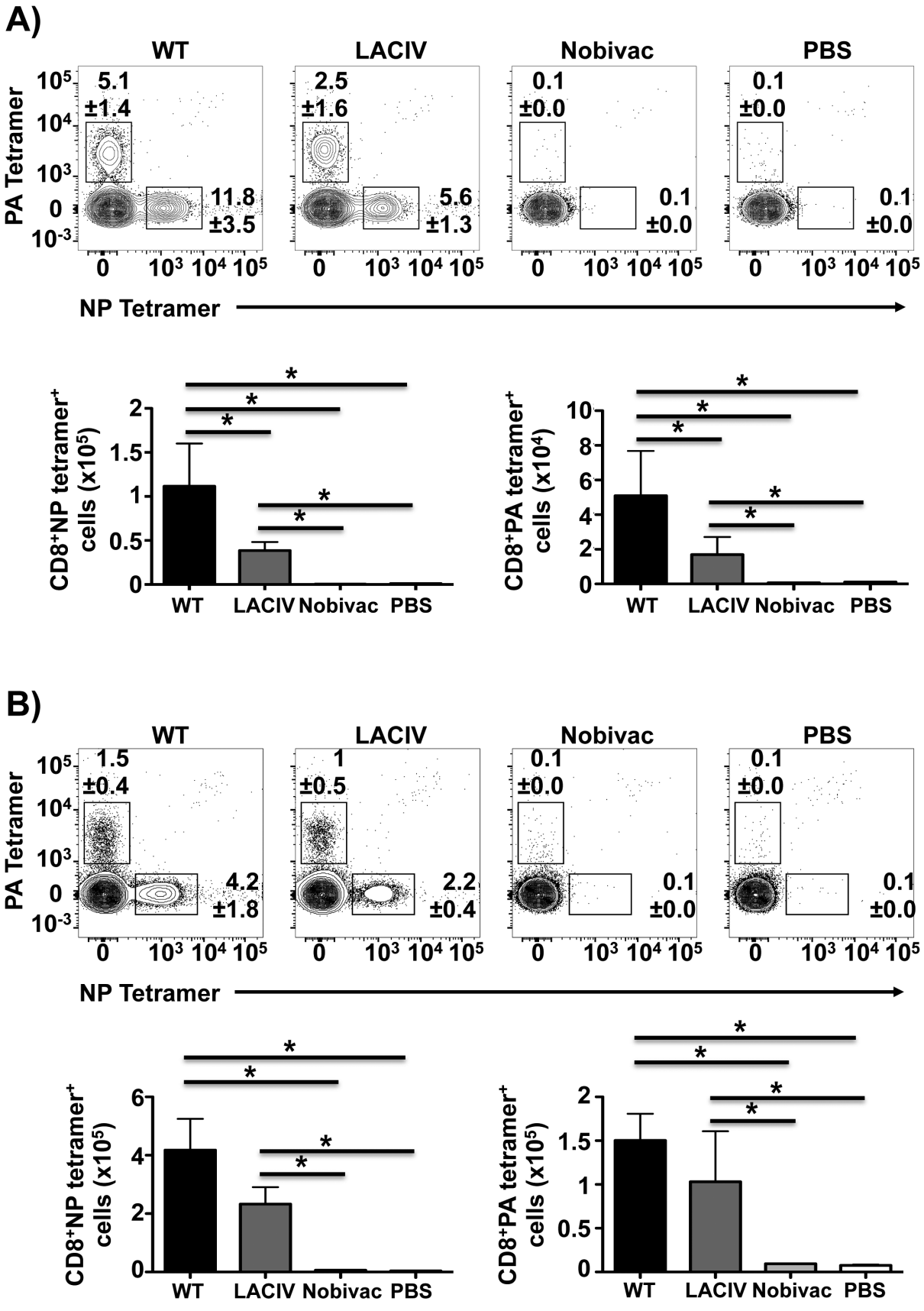












Journal of Virology